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Crop Protection 18 (1999) 531–537

Crop Protection

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Parasitism of diapausing pink bollworm *Pectinophora gossypiella* (Lepidoptera: Gelechiidae) larvae by entomopathogenic nematodes (Nematoda: Steinernematidae, Heterorhabditidae)☆

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Received 13 May 1999; received in revised form 9 July 1999; accepted 12 July 1999

Abstract

Diapausing larvae of *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), were exposed to entomopathogenic nematodes (Nematoda: Steinernematidae; Heterorhabditidae) at a dosage of 100 or 200 infective juveniles (IJ) per larva. No significant differences between infection of *Steinernema riobrave* (strain 355) Cabanillas, Poinar and Raulston, and *Heterorhabditis bacteriophora* (strain Cruiser) (Poinar) were observed after either 6-d or 9-d of incubation. No significant difference in infection levels occurred due to increasing dose. Greatest insect mortality occurred due to parasitism by *S. riobrave* (355). In a second assay, diapausing larvae of *P. gossypiella* were exposed to *S. riobrave* (355), *Steinernema carpocapsae* (strain Kapow) (Weiser), *H. bacteriophora* (strain Lawn Patrol), or *H. bacteriophora* (Cruiser) at dosages of 50, 100, 200, or 400 IJs per larva. *S. carpocapsae* and *H. bacteriophora* (Cruiser) infected larvae at significantly higher rates compared with *H. bacteriophora* (Lawn Patrol) and *S. riobrave*. A significant difference in infection levels occurred due to nematode dose. In a third assay cotton bolls infested with diapausing *P. gossypiella* were exposed to *S. riobrave* (355), or *H. bacteriophora* (Cruiser). Boxes were prepared with bolls buried 5 cm below the soil surface or bolls laid on the soil surface. Boxes also were incubated at constant temperature or exposed to ambient seasonal temperatures and light. *H. bacteriophora* (Cruiser) infected larvae at significantly higher rates compared with *S. riobrave* (355). Significant differences in infection levels occurred due to the location of the boll placement and the incubation temperature.

Keywords: Entomopathogenic nematodes; Infectivity; Temperature; *Steinernema*; *Heterorhabditis*; *Pectinophora gossypiella*; Insect diapause

1. Introduction

Steinernematidae and Heterorhabditidae (Rhabditida) are entomopathogenic nematodes that are effective against a wide range of soil-inhabiting insects (Begley, 1990; Klein, 1990). Developments in production of these biocontrol agents through liquid fermentation (Georgis, 1990), expansion of the number of in vivo producers and the exemption from registration requirements in most countries (Gaugler, 1988) have favored their commercial development (Georgis, 1992). Entomopathogenic nematology has been a growing research emphasis for over five decades (Smith et al., 1992; Webster, 1998). These efforts have led to the successful introduction of many products into a variety of markets (Georgis and Hague, 1991).

Steinernematids and heterorhabditids are obligate insect parasites (Poinar, 1979) with associated bacterial symbionts, *Xenorhabdus* spp. and *Photorhabdus* spp., respectively (Akhurst and Boemare, 1990). The infective juvenile (IJ) stage of the nematode remains in the soil until it can invade the body of a susceptible insect. After infection, the symbiotic bacteria are released into the insect hemocoel, causing septicemia and death of the insect (Kaya and Gaugler, 1993). Nematodes feed on the bacterial cells and certain components of host tissues. Nematodes may pass through several generations in an individual insect. Once host reserves are depleted, a new generation of IJs exit the cadaver (Kung et al., 1991).

Research has indicated that several lepidopteran cotton pests, including *Helicoverpa zea* (Boddie), *Spodoptera*

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0261-2194/99/$ - see front matter
PII: S0261-2194(99)00057-5

frugiperda (Smith) (Raulston et al., 1992), *Spodoptera exigua* (Hübner), *Trichoplusia ni* (Hübner), and *Pectinophora gossypiella* (Saunders) (Henneberry et al., 1995; Gouge et al., 1996, 1997), are susceptible to *Steinernema* spp. Typically, the larval and pupal stages of each lepidopteran species has been tested for nematode susceptibility.

The ability of pink bollworm (PBW), *P. gossypiella*, to enter diapause before the seasonal onset of unfavorable environmental conditions greatly enhances its survival and therefore increases its damage potential the following season. Late instar PBW cut out of the boll and drop to the ground to enter diapause in the top few centimeters of soil, or remain in the bolls and diapause near or within the seeds. Larvae spin a silk cocoon in either case (University of California, 1984).

The hormonal control of morphogenesis changes as a result of photoperiod (Adkisson, 1961), nutrition (Foster and Crowder, 1980; Raina and Bell, 1978), temperature (Bull and Adkisson, 1960), and even drought (Fife, 1949). Diapause is restricted to the pre-pupal PBW stage but it is possible that earlier stages act as the recipient of the environmental signals initiating the delay in development (Chapman, 1971). As a result, the insect is able to increase its nutritional reserves and become dormant before environmental conditions become limiting. Insect metabolic rate is very low during diapause and biochemical changes occur at low temperatures, ultimately leading to reactivation of the humoral system so that growth and development is resumed (Chapman, 1971). Reduced metabolic rate together with biochemical changes could render a potential insect host less attractive to entomopathogenic nematodes.

This study investigates the possibility of controlling diapausing PBW populations using entomopathogenic nematodes. Specifically, we investigated the effect of incubation period, nematode species, and nematode dose on the infection and mortality of diapausing PBW. Additionally, we considered nematode control of diapausing and non-diapausing PBW that remain within the cotton boll.

2. Materials and methods

2.1. Nematode sources

Steinernema riobrave (strain 355) Cabanillas, Poinar and Raulston, were obtained from a commercial sample formulated in water dispersible granules, produced by Biosys Inc. (Columbia, MD). The strain was originally isolated from soil samples collected in the Lower Rio Grande Valley, TX. *Steinernema carpocapsae* (strain Kapow) (Weiser), had been maintained in culture at Western Cotton Research Laboratory, Phoenix, AZ for 5 years and was originally isolated from Chihuahua, Mexico. *Heterorhabditis bacteriophora* (strain Lawn Patrol) (Poinar), was cultured from commercial samples formulated on sponge by Hydro Gardens (Colorado Springs, CO). *Heterorhabditis bacteriophora* (strain Cruiser) was cultured from a commercial sample, formulated in a clay-based substrate by Ecogen (Langhorne, PA).

Infective juvenile (IJ) entomopathogenic nematodes were obtained by in vivo culturing (Kaya and Stock, 1997) in late instar *Galleria mellonella* L. *G. mellonella* larvae were infected at 27°C for *S. riobrave* and *H. bacteriophora*, and 22°C for *S. carpocapsae*. Infective juveniles were extracted using White traps (White, 1927), and stored in distilled water at 15°C (*S. riobrave* and *H. bacteriophora*), or 7°C (*S. carpocapsae*). Nematodes were stored for no longer than 4 d prior to use.

2.2. Insect sources

Green cotton bolls (Pima S-6, *Gossypium barbadense* L.) were collected prior to harvest from heavily infested fields. The bolls were maintained at 16°C, with an 8-h photophase. Pink bollworm larvae were collected from naturally infested bolls by dissecting the bolls and gently removing the insects. Larvae were not removed from their silk cocoons. Diapause was determined by placing larvae in the diapause-averting conditions of 30°C, with a 13-h photophase for 7 d. Diapause was then evidenced by failure to pupae.

2.3. Effect of incubation period on infection and mortality of diapausing pink bollworm

Individual diapausing late instar PBW were placed on the surface of sterile sand (sieved through US mesh size 50, 10% moisture, weight/weight) in 3.5 cm diameter petri dishes. IJ nematodes of *S. riobrave* or *H. bacteriophora* (Cruiser) were acclimated at 22°C for 4 h, and then pipetted onto the sand surface in 0.5 ml of distilled water, at a dose of 0, 100 or 200 IJs per Petri dish. Seven replicate petri dishes were made for each of two incubation periods of 6 and 9 d. Dishes were sealed with Parafilm® and incubated at 17°C, with an 8-h photophase. After incubation, insects were rinsed three times in distilled water and placed in clean Petri dishes lined with moist filter paper and sealed with Parafilm®. The dishes were incubated at 27°C for an additional 3 d. Insect mortality was recorded and the number of nematodes present within each larva was counted by dissecting individual larvae in quarter strength Ringer's solution, under a stereo microscope.

2.4. Effect of dose rate and nematode species on infection of diapausing pink bollworm

Individual late instars were placed on the surface of sterile sand (10% moisture by weight) in 3.5-cm diameter

Petri dishes. IJ nematodes of *S. riobrave*, *H. bacteriophora* (Cruiser), *H. bacteriophora* (Lawn Patrol), or *S. carpocapsae* were acclimated and pipetted onto the sand surface in 0.5 ml of distilled water, at doses of 0, 50, 100, 200, or 400 IJs per Petri dish. Seven replicate Petri dishes were made for each of the four nematodes. Dishes were sealed with Parafilm® and incubated for 7 d at 16°C, with an 8-h photophase. After incubation, insects were rinsed three times in distilled water and placed in clean Petri dishes at 27°C for an additional 3 d. Insects were washed and dissected as before. The number of nematodes present within each larva was counted.

2.5. Infection of diapausing and non-diapausing pink bollworm in green bolls

Diapause of pink bollworm larvae within bolls could not be confirmed prior to nematode application. Pink bollworm-infested bolls (*G. barbadense*) were incubated in plastic boxes (20 cm × 30 cm × 8 cm) filled with sterile potting soil. Boxes were prepared with bolls either buried 5 cm below the soil surface or laid on the soil surface. IJs of *S. riobrave* (355) or *H. bacteriophora* (Cruiser) were applied to the boxes at 20,000 IJs (2.5 billion per ha) per box containing 25 bolls. Half the boxes were incubated in a constant temperature incubator, and half the boxes were incubated outside, exposed to ambient seasonal temperatures. The experiment was conducted during October and then repeated in December. The October (fall) test involved a constant temperature incubation of 27°C, 10 : 14 (light : dark), and ambient field incubation at 17–35°C at the soil surface and 21–27°C 5 cm below the soil surface, and ambient light conditions (11.8 : 12.2 hours, light : dark). The December (winter) test involved a constant temperature incubation of 16°C, 8 : 16 (light : dark), and an ambient field incubation of 15–21°C at the soil surface and 13–18°C 5 cm below the soil surface, and ambient light conditions (9.7 : 14.3 h, light : dark). Five replicate boxes were constructed for each treatment combination. Boxes were weighed periodically and moisture levels were maintained at 15% ($\pm$ 5%). After 5 d the bolls were broken open, insects removed and dissected. The number of infecting nematodes and insect mortality was recorded.

Factorial analysis of variance was used to test for significant differences among treatment means. The Student–Newman–Keuls test was used to partition means into significant ranges when a significant F value was determined by analysis of variance. Mortality data were transformed using an arc sine transformation prior to analysis. Simple linear regression analysis was used to establish a constant change hypothesis between number of nematodes applied and number of nematodes infecting. The 5% level of probability was used in all statistical tests. The statistical software program CoStat (1995) was used for all statistical analysis.

3. Results

3.1. Effect of incubation period on infection and mortality of diapausing pink bollworm

No infection or insect mortality occurred in control treatments receiving water only. No significant difference in the number of nematodes infecting occurred due to increasing the dose of entomopathogenic nematodes from 100 to 200 IJs per diapausing pink bollworm larva ($F = 0.97$; df = 1, 55; $P = 0.330$) (Fig. 1). Additional infection by both nematode species occurred during the longer 17°C incubation period ($F = 13.51$; df = 1, 55; $P = 0.001$, Fig. 1). No interaction between nematode species and dose was observed ($F = 1.12$; df = 1, 55; $P = 0.293$). No significant differences between infection by *S. riobrave* or *H. bacteriophora* (Cruiser) were observed after either 6-d ($F = 2.73$; df = 1, 27; $P = 0.111$) or 9-d incubation ($F = 1.91$; df = 1, 27; $P = 0.180$). The numerically highest insect mortality occurred due to parasitism by *S. riobrave* (Fig. 2).

3.2. Effect of dose rate and nematode species on infection of diapausing pink bollworm

No infection occurred in control treatments receiving water only. *S. carpocapsae* and *H. bacteriophora* (Cruiser) infected diapausing pink bollworm larvae at significantly higher rates compared to *H. bacteriophora* (Lawn Patrol) and *S. riobrave* ($F = 19.26$; df = 3, 111; $P < 0.001$, Fig. 3). A significant difference in infection levels occurred due to nematode dose ($F = 6.59$; df = 3, 111; $P < 0.001$, Fig. 3). No interaction between nematode species and dose was observed ($F = 0.66$; df = 1, 111; $P = 0.735$).

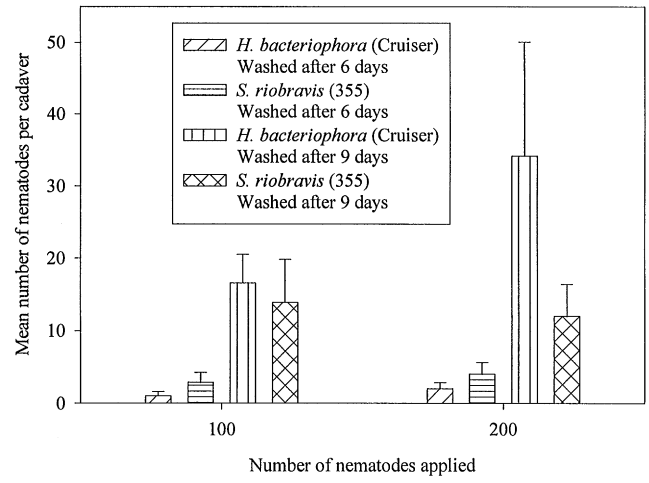

Fig. 1. Mean number of *Steinernema riobrave* (355) and *Heterorhabditis bacteriophora* (Cruiser) infecting diapausing *Pectinophora gossypiella*. Nematodes were applied at 100 or 200 infective juveniles per insect, and incubated at 17°C for 6 or 9 d. Bars indicate standard errors of means (n = 7).

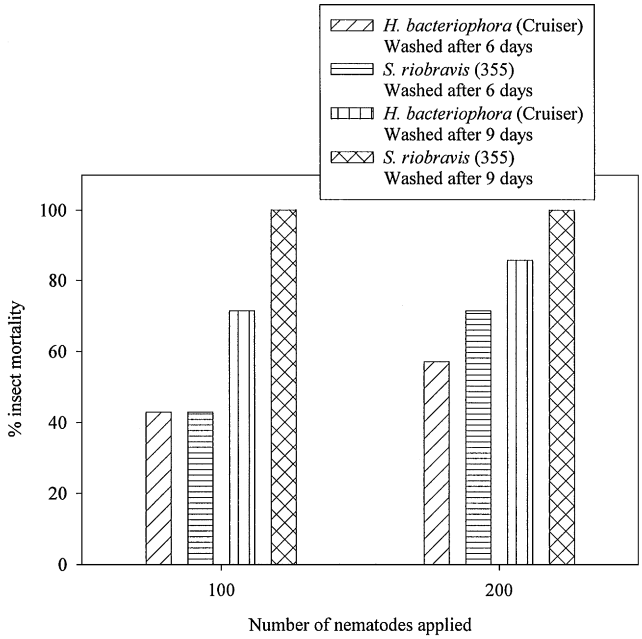

Fig. 2. Mortality of diapausing *Pectinophora gossypiella*. Nematodes were applied at 100 or 200 infective juveniles per insect, and incubated at 17°C for 6 or 9 d.

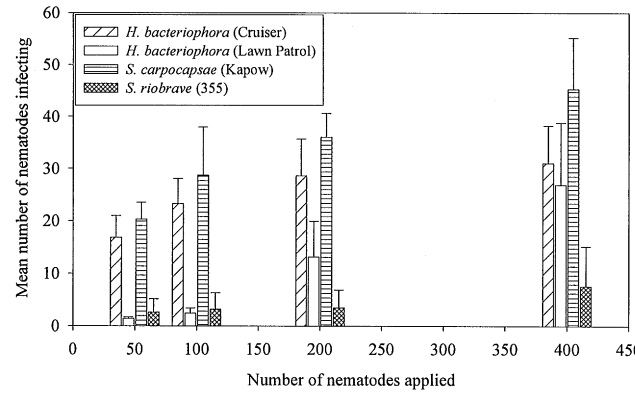

Fig. 3. Mean number of *Steinernema riobrave* (355), *Heterorhabditis bacteriophora* (Cruiser), *Heterorhabditis bacteriophora* (Lawn Patrol), and *Steinernema carpocapsae* (Kapow) infecting diapausing *Pectinophora gossypiella*. Nematodes were applied at 50, 100, 200, or 400 infective juveniles per insect, and incubated at 16°C for 7 d. Bars indicate standard errors of means.

The relationship between nematode species, dose, and the number of nematodes infecting diapausing larvae could be described using quadratic regression analyses ($y = y_0 + ax + bx^2$). *H. bacteriophora* (Cruiser), $R^2 = 0.987$ ($y = 11.585 + 0.126x \pm 1.95 \times 10^{-4}x^2$), *H. bacteriophora* (Lawn Patrol), $R^2 = 0.986$ ($y = -3.808 + 0.083x \pm 1.46 \times 10^{-5}x^2$), *S. carpocapsae*, $R^2 = 0.990$ ($y = 14.16 + 0.148x + -1.77 \times {}^{10^{-4}}x^2$), *S. riobrave*, $R^2 = 0.990$ ($y = 2.877 \pm 4.572 \times 10^{-3}x + 4.062 \times 10^{-5}x^2$).

3.3. Infection of diapausing and non-diapausing pink bollworm in green bolls

During the December (winter) experiment no significant differences in infection occurred due to nematode species ($F = 1.76$; df = 1, 53; $P = 0.191$), boll placement at the surface or 5 cm below ($F = 1.76$; df = 1, 53; $P = 0.191$), or incubation in the field or laboratory ($F = 0.26$; df = 1, 53; $P = 0.612$). However, during the fall experimental period *H. bacteriophora* (Cruiser) infected larvae at significantly higher rates compared with *S. riobrave* ($F = 35.69$; df = 1, 53; $P < 0.001$, Table 1). Also during the fall experiment, significant differences in infection levels occurred due to incubation in the field or laboratory ($F = 4.38$; df = 1, 53; $P = 0.037$), with larger numbers of nematodes infected larvae incubated in the laboratory at 27°C (Table 1). Boll placement at the surface or 5 cm below the surface did not influence nematode infection significantly during the fall period ($F = 3.75$; df = 1, 53; $P = 0.054$, Table 1).

A significant interaction occurred between nematode species and incubation placement in the field or constant temperature laboratory incubation ($F = 4.38$; df = 1, 53; $P = 0.037$).

S. riobrave failed to infect any pink bollworm contained within the cotton bolls (Table 1). *H. bacteriophora* (Cruiser) infected larvae within bolls in low numbers (means < 0.83 IJs per insect, Table 1). Resulting mortality of pink bollworm contained in cotton bolls, caused by *H. bacteriophora* (Cruiser) did not exceed 20% (Table 1). No significant effects due to boll placement at the surface or 5 cm below the surface ($F = 2.39$; df = 1, 39; $P = 0.132$) were found. Similarly, no significant effects were apparent due to incubation in the field versus the constant temperature laboratory incubation ($F = 0.01$; df = 1, 39; $P = 0.917$). However, there were significant differences in mortality levels due to fall or winter season in which the experiment was conducted ($F = 5.37$; df = 1, 39; $P = 0.027$).

4. Discussion

Insect susceptibility to entomopathogenic nematodes in the families Steinernematidae and Heterorhabditidae is dependent upon nematode species and is profoundly affected by temperature (Molyneux et al., 1984; Molyneux, 1985; Gaugler, 1988; Kaya, 1990; Grewal et al., 1994). Temperature tolerance varies among nematode species and strains (Molyneux, 1986; Griffin and Downes, 1991; Grewal et al., 1993, 1994). Several nematode species are reported to be highly infective at low temperatures (9–12°C) (Griffin and Downes, 1994, 1991).

Molyneux et al. (1984) concluded that variability in nematode infectivity in different hosts is, in part, related

Table 1
Mean number of *Heterorhabditis bacteriophora* (Cruiser) and *Steinernema riobrave* (355), infecting insects and mortality of *Pectinophora gossypiella*. Nematodes were applied to infested bolls at 2.5 billion per ha and incubated for 5 d in a constant temperature incubator or in the field. Infested bolls were laid either at the soil surface or at a depth of 5 cm

			Mean number of nematodes		% mortality	
		Incubation temperature (°C)	*H. bacteriophora* (Cruiser)	*S. riobrave* (355)	*H. bacteriophora* (Cruiser)	*S. riobrave* (355)
Winter-diapausing larvae	Incubator	0 cm soil depth-Constant 16°	0	0	0	0
		5 cm soil depth-Constant 16°	0.5 (0.5)	0	16.67	0
	Field	0 cm soil depth- 15–21°	0	0	0	0
		5 cm soil depth- 13–18°	0.22 (0.22)	0	11.11	0
Fall-late instar larvae	Incubator	0 cm soil depth-Constant 27°	0.32 (0.16)	0	0	0
		5 cm soil depth-Constant 27°	0.83 (0.22)	0	20.00	0
	Field	0 cm soil depth- 17–35°	0.26 (0.14)	0	0	0
		5 cm soil depth- 13–18°	0.29 (0.10)	0	13.33	0

Standard errors of means are indicated in brackets; The experiment was conducted during fall and winter months.

to the length of time the insect host remains susceptible to nematode parasitization at different temperatures. Significant differences in the levels of nematode infection due to length of incubation at 16°C indicate that less cold tolerant nematodes such as *S. riobrave* may still be useful as biocontrol agents, assuming that the nematodes are capable of persisting for longer in an infective state.

S. carpocapsae and *H. bacteriophora* (Cruiser) infected diapausing larvae at higher rates compared with *H. bacteriophora* (Lawn Patrol) and *S. riobrave*. Both of the former nematode species are known to be active at 16°C (Griffin and Downes, 1991; Kung et al., 1991). Several *H. bacteriophora* (Poinar) strains that have been isolated, have been shown to have a range of different temperature preferences (Glazer et al., 1996; Grewal et al., 1994).

A linear relationship between the number of nematodes applied to insects and the numbers of nematodes infecting has been established for several insect species (Fan and Hominick, 1991). However, it may be expected that infection of an insect in diapause could depend on different factors that may not result in a linear response between dose and infection level. Entomopathogenic nematodes respond to chemical insect cues such as carbon dioxide (Gaugler et al., 1980) and a variety of nitrogenous chemical gradients (Schmidt and All, 1979). Byers and Poinar (1982) established that some nematode species also respond to heat conducted from insect larvae. Insect metabolism is often measured using the respiration quotient (CO_2 output/O_2 input), and generally, a decrease in ambient temperature will cause a fall in insect metabolic rate (Edwards, 1953). At lower temperatures, an insect in diapause (with a depressed metabolism) likely releases relatively few attractant factors. Our studies show that infection of diapausing pink bollworm is better described as quadratic relative to the number of nematodes applied.

Small proportions of PBW do not exit but diapause within the cotton bolls. Our final experiment considered the possibility of nematodes entering the mines made by the neonate larvae, or other apertures, and moving through the bolls to infect the insects. Harris et al. (1990), describe the successful control of a leaf mining species *Liriomyza trifolii* (Burgess) using *S. carpocapsae*. However, *S. riobrave* failed to locate pink bollworm within the cotton bolls during either fall or winter experiments. *H. bacteriophora* (Cruiser) infected insects at both soil depths and in both incubation regimes during the fall experiment. During the fall tests a large proportion of the pink bollworm larvae broke diapause and began to develop normally. *H. bacteriophora* (Cruiser) did infect a small number of larvae during the winter period but all infected PBW were extracted from bolls which had been at a depth of 5 cm. As the temperatures were equivalent for 0 and 5-cm depths during constant temperature incubation of 27°C, the difference in nematode infection levels must be due to other reasons such as positive geotropism (Duncan et al., 1996; Hanula, 1993) or more suitable moisture levels (Kondo and Ishibashi, 1985; Kung et al., 1991). A significant interaction occurred between nematode species and incubation placement in the field or constant temperature laboratory incubation, reconfirming the knowledge that the efficacy of different species of nematodes differs under different abiotic and biotic conditions.

H. bacteriophora infected pink bollworm in very low numbers (mean IJs < 0.32 per insect) during the fall experiment at 0 cm soil depth but did not cause any insect mortality. The nematodes infecting insects in these

cases were always in the juvenile form. It may be assumed that they were individuals that had infected very recently. It is possible that decreased moisture levels at the surface of the soil delayed nematode progress. It can be assumed that insects infected with nematodes would die eventually.

H. bacteriophora (Cruiser) appeared to have greater cold tolerance compared to *S. riobrave*. Neither of the nematode species tested is suitable for the control of pink bollworm while the insect remains within the cotton bolls. However, larvae diapausing in soil are susceptible even during winter conditions.

During the winter experiment the key influencing factor regarding nematode infection was boll positioning. However, during the fall experiment it was temperature that influenced nematode infection to a greater degree.

Acknowledgements

We thank Kirk A. Smith, Darcy Reed, and Nilima Prabhaker for critical review of this manuscript.

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